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Orexin A Stimulates Hypothalamic–Pituitary–Adrenal (HPA) Axis Function, but not Food Intake, in the Absence of Full Hypothalamic NPY-ergic Activity

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Neonatal monosodium L-glutamate (MSG) treatment destroys hypothalamic arcuate nucleus neuronal bodies, thus inducing several metabolic abnormalities. As a result, rats develop a phenotype characterized by hyperleptinemia and by impaired NPY but normal prepro-orexin hypothalamic mRNAs expression. Thus, our study was designed to explore whether hypothalamic effects of orexin A on food intake and glucocorticoid production develop in the absence of full hypothalamic NPY-ergic activity. For this purpose we evaluated, in control and MSG-treated rats, the consequences of intracerebroventricular (icv) orexin A administration on food intake and changes in circulating levels of ACTH and glucocorticoid. Our results indicate that orexin A icv treatment stimulated hypothalamic–pituitary–adrenal (HPA) axis activity in both MSG-damaged and normal animals, with this response even more pronounced in neurotoxin-damaged rats. Conversely, food intake was only enhanced by icv orexin A injection in normal rats. Our study further supports that acute hypothalamic effects of orexin A on food intake and glucocorticoid production are due to independent neuronal systems. While intact arcuate nucleus activity is needed for the orexinergic effect induced by icv orexin A administration, conversely, orexin A–stimulated HPA axis function takes place even in the absence of full NPY-ergic activity.

Key Words: Hypothalamic obesity; food intake; MSG; orexinergic pathways; stress hormones; leptin.

Introduction

The hypothalamic control of food intake and energy expenditure pertains to the complex neural circuitry regulating appetite/satiety *(1)*. The peripheral–hypothalamic interaction of orexigenic and anorexigenic signals is responsible for, among other functions, maintaining homeostasis. It is openly recognized that hypothalamic NPY is the most relevant physiological hunger signal *(2,3)*. However, other neural mechanisms are also involved in the normal activity of the hypothalamic circuit controlling food intake *(1)*. Among them, orexin A is an important signal regulating feeding behavior *(4)*. It has been proposed that orexin A impacts hypothalamic arcuate nucleus (ARC) NPY neuronal cell bodies *(5)* through the activation of specific orexin A–receptor 1 *(6,7)*. Recently, it has been claimed that orexin A neurons are responsible for the ghrelin-induced food intake by communicating, as an interneuronal system, the ghrelin signal and NPY-ergic activity *(8)*. However, other authors reported that orexin A–stimulated food intake can only be developed after decreasing the anorexigenic corticotrophic-releasing hormone (CRH) activity *(9)*.

It is accepted that orexin A also modulates the hypothalamic-pituitary–adrenal (HPA) axis function *(10–13)*, thus cooperating through this pathway to maintain homeostasis *(14)*. Regarding the mechanism of orexin A action on HPA axis function, it has been established that orexin A is able to release CRH from hypothalamic tissues, an effect abrogated by NPY Y1 receptor antagonist *(11)*. However, other studies indicate that the capability of orexin A, intracerebroventricular (icv) administered, to stimulate HPA axis function is due to the direct activation of parvo- and magnocellular neurons of the hypothalamic paraventricular nucleus (PVN) *(12)*, thus indicating that whether or not the participation of the hypothalamic NPY-ergic system in the orexin A–stimulated HPA axis activity still remains unclear.

The neonatal treatment of rats with monosodium L-glutamate (MSG) induces a phenotype characterized by several neuroendocrine and metabolic alterations, such as reduced hypothalamic expression of NPY mRNA *(15)*, hypophagia *(15,16)*, hyperadiposity *(15,16)*, HPA axis hyperresponse *(15,17)* and insulin resistance *(18)*, among others. It is recognized that this neurotoxic compound mainly affects ARC neuron cell bodies *(19–21)*.

The aim of the present study was to test the hypothesis that while the stimulatory effect of orexin A on food intake could be developed via a full NPY-ergic activity, orexin-A–

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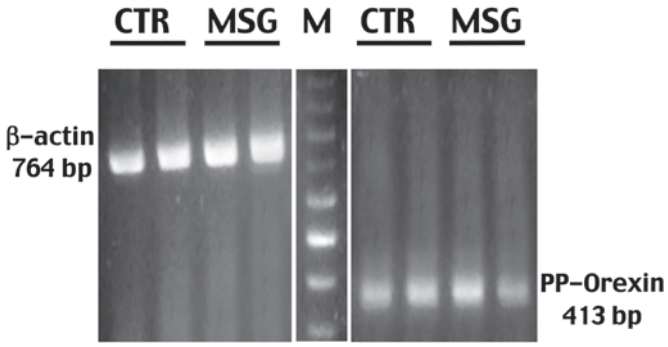

Fig. 1. RT-PCR expression analysis of hypothalamic prepro (PP)-orexin mRNA in control and MSG-damaged female rats (one representative experiment, two individuals per group) (M: molecular marker, 100-bp ladder).

stimulated HPA axis function could remain after the damage of the ARC. For this purpose, orexin A icv administration was performed in normal and MSG-damaged rats, and both food intake and HPA axis activity were evaluated.

Results

Hypthalamic NPY and Prepro-Orexin Expression in 120-d-old Normal (CTR) and ARC-Damaged (MSG) Female Rats

Preliminary experiments were performed in order to assess the effectiveness of the damage, induced by neonatal MSG treatment, on hypothalamic NPY mRNA expression. The results confirm our previous data *(15)* and indicate that the neonatal administration of the neurotoxic compound did reduce, as compared to normal rats, hypothalamic NPY mRNA expression by approx 60% (in arbitrary units and relative to β-actin mRNA expression: 0.868 ± 0.043 in CTR rats, and 0.368 ± 0.075 in MSG animals; $n = 6–8$ rats per group, $p < 0.05$). In the same tissues, prepro-orexin mRNA expression was also evaluated. As depicted in Fig. 1, prepro-orexin mRNA expression was similar in both groups examined. In fact, hypothalamic prepro-orexin mRNA expression was not changed as a consequence of MSG treatment (in arbitrary units and relative to β-actin mRNA expression: 0.321 ± 0.013 in CTR rats and 0.368 ± 0.077 in MSG animals; $n = 6–8$ rats per group).

HPA Axis and Food Intake Responses to icv Administration of Orexin A in Ad Libitum *Eating, Normal (CTR), and Hyperleptinemic (MSG) Female Rats*

This experiment was designed to evaluate whether icv administration of orexin A is able to modify food intake and HPA axis function in CTR and MSG rats eating *ad libitum*. When rats from both groups were icv administered with either vehicle alone or containing 1 μg orexin A, we found that 3 h after treatment while CTR rats respond to the treatment by significantly ($p < 0.05$ vs vehicle-treated animals) enhancing 3-h-food intake (3.88 ± 1.08 and 1.9 ± 0.89 g per rat, in orexin A and vehicle injected rats, respectively), conversely no differences were found in the amount of food eaten (during 3 h) by MSG rats treated (0.91 ± 0.32) or not (0.87 ± 0.33) with orexin A. It should be noted that, after vehicle administration, MSG rats were significantly ($p < 0.05$) hypophagic compared to CTR rats. It should be noted that although individual MSG (174.6 ± 10.8 g BW) are significantly ($p < 0.05$) lighter than CTR (223.4 ± 7.48 g BW) rats, they developed a very significant increase in fat mass (data not shown, see ref. *15*). Thus, the hypophagia characterizing MSG-lesioned rats could be related to their low lean body mass.

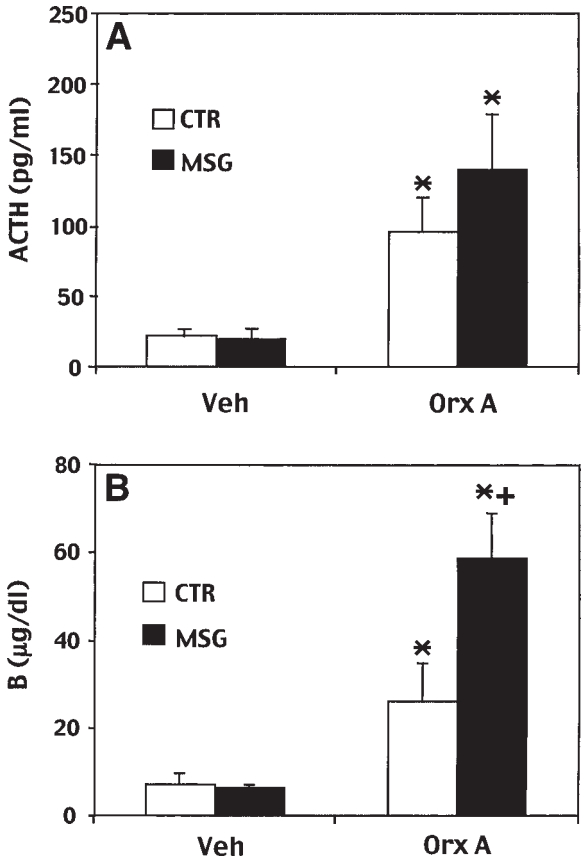

Fig. 2. Circulating levels of ACTH (panel **A**) and corticosterone (panel **B**) 3 h after icv administration of 2 μL of vehicle alone (Veh) or containing 1 μg of orexin A (Orx A) in *ad libitum* eating, CTR, and MSG rats. Experimentation was performed at 09:00 hours. Values are the mean ± SEM, $n = 6–8$ rats per group. $^{*}p < 0.05$ vs values in Veh-injected rats. $^{+}p < 0.05$ vs values in MSG rats in similar condition.

Figure 2 shows the results of circulating levels of ACTH (panel A) and corticosterone (panel B) in CTR and MSG, eating *ad libitum*, 3 h after icv administration of vehicle or orexin A. As depicted, both groups of rats developed corticotroph and adrenal responses to orexin A administration, when measured 3 h after treatment. Although corticotroph responses did not differ between groups (panel A), MSG animals displayed significantly ($p < 0.05$) higher circulating glucocorticoid levels than CTR rats (panel B).

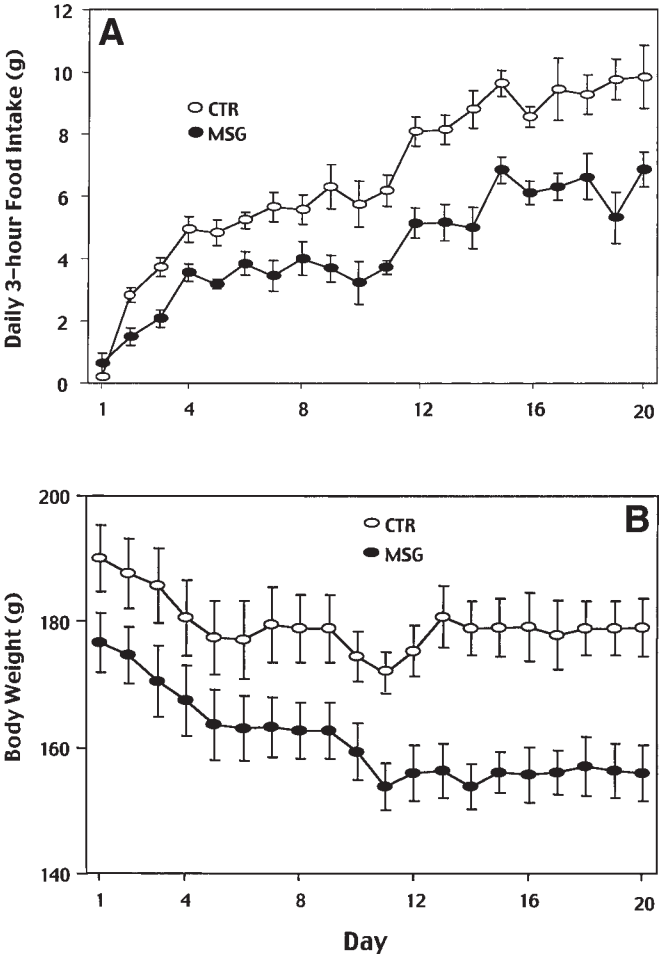

Fig. 3. Daily 3-h food intake (panel **A**) and changes in body weight values (panel **B**), between d 1 and 20 of the food restriction protocol, in normal (CTR) and hypothalamic-damaged (MSG) female rats. Values are the mean ± SEM, $n = 16–20$ rats per group. All CTR values, except food intake on d 1, were significantly ($p < 0.05$) higher than MSG values.

Influences of Food Restriction on Changes in Body Weight and Leptinemia in CTR and MSG Female Rats

Because MSG rats are hyperleptinemic, we submitted CTR and MSG to the food-restriction protocol in order, once stabilized their body weights, to decrease leptin circulating levels that could mask any orexigenic effect of orexin A.

Figure 3 shows the results of daily 3-h food intake and BW values, in CTR and MSG rats, between d 1 and 20 of the food-restriction paradigm. As depicted, except for d 1, MSG rats under food restriction regime did eat a significantly ($p < 0.05$) less amount of food, during the 3-h period of food allowance, than their normal counterparts (CTR), regardless of the experimental day examined (panel A).

Changes in BW values induced by this food-restriction protocol indicated that BW values decreased in both experimental groups during the first 12 d of food restriction, and that the low BW displayed by MSG on d 1 held through the last day recorded (panel B). Interestingly, BW values were stabilized from d 13 to 20, regardless of the group examined.

Table 1
Plasma Leptin Concentrations (ng/mL) in CTR and MSG Rats Either Eating *Ad Libitum* (AL) or on d 21 After Eating According to the Food-Restriction (FR) Protocol[a]

Group/condition	CTR	MSG
AL	4.97 ± 1.24	19.11 ± 3.32^{+}
FR	0.72 ± 0.51*	2.75 ± 0.78^{+}*

$^{+}p < 0.05$ vs CTR values.
$^{*}p < 0.05$ vs AL values in the same group.
[a]Values are the mean ± SEM ($n = 6–8$ rats per group).

Table 1 shows the results of circulating leptin levels, in rats from both groups, obtained after animals either were or were not (for comparison purposes) subjected to the food-restriction protocol. As it can be seen, in *ad libitum* eating rats, a clear hyperleptinemia characterized MSG animals. On d 21 after food restriction, circulating leptin concentrations significantly decreased (vs respective group, *ad libitum* values) in both groups of rats. It is interesting that plasma leptin levels in MSG rats on this day were of a similar magnitude to those in CTR rats eating *ad libitum*.

Activation of HPA Axis Function by Orexin A icv Administration in Food-Restricted, CTR, and MSG Rats

Figure 4 shows the results of circulating ACTH (panels A and C) and corticosterone (panels B and D) concentrations in the 3-h period following 1 μg icv administration of orexin A in CTR and MSG rats subjected to 21 d of food intake restriction. Injection (icv) of vehicle alone did not modify basal circulating ACTH and B levels, regardless of time and group examined (Fig. 4, panels A–D). As depicted, orexin A administration did increase plasma ACTH concentrations over the baseline in both groups of rats (Fig. 4, panels A and C); however, maximal pituitary responses were higher in MSG than in CTR rats (see below). The increase in ACTH secretion, following orexin A treatment, in turn enhanced circulating glucocorticoid levels over the respective baselines in both experimental groups (Fig. 4, panels B and D); maximal adrenal responses were higher in MSG than in CTR rats (see below). Importantly, the resilience of the adrenal response was somewhat delayed in MSG vs CTR rats (see Fig. 4, panels B and D).

When the areas under the curves (AUCs) for circulating ACTH and B concentrations (in the 3-h period examined) were calculated (Table 2), significantly ($p < 0.05$) higher values for both metabolites were found in MSG than in CTR rats.

Effect of Orexin A icv Treatment on 3-h Food Intake in Food-Restricted, CTR, and MSG-Damaged Rats

Food intake for 3 h following icv treatment with either vehicle alone or containing orexin A is depicted in Fig. 5.

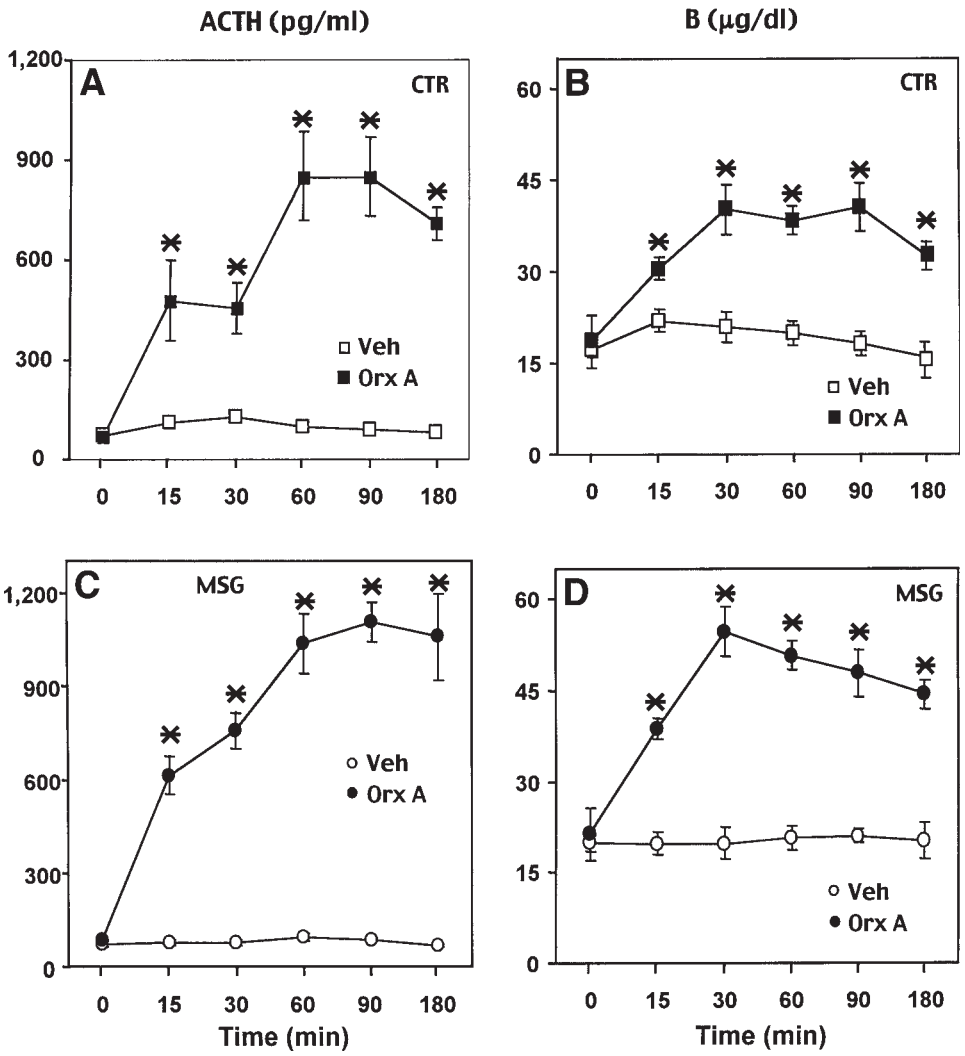

Fig. 4. Circulating levels of ACTH (panels **A** and **C**) and corticosterone (panels **B** and **D**) before and several times after icv administration of 2 µL of vehicle alone (Veh) or containing 1 µg of orexin A (Orx A) in CTR (panels **A** and **B**) and MSG (panels **C** and **D**) rats. Experimentation was performed at 09:00 hours on d 21 of the food-restriction protocol. After rats were bled at time zero, they had free access to food for 3 h. Values are the mean ± SEM, n = 6–8 rats per group. *$p < 0.05$ vs respective time-values in Veh-injected rats (ANOVA with repeated measurement).

Table 2
Integrated 3-h Circulating ACTH and Corticosterone After icv Administration of 1 µg of Orexin A, in Food-Restricted, CTR, and MSG Rats[a]

Group	CTR	MSG
ACTH (pg/mL/3 h)	2871.27 ± 208.84	4108.02 ± 305.17[+]
Corticosterone (µg/dL/3 h)	74.16 ± 4.59	108.97 ± 7.91[+]

[+]$p < 0.05$ vs CTR values.
[a]Calculated values from Fig. 4. Values are the mean ± SEM, n = 6–8 rats per group.

On d 21 of the food-restriction protocol, MSG animals were, as expected, hypophagic (vs CTR rats) after treatment with vehicle alone. As shown, orexin A administration was able to significantly ($p < 0.05$) enhance 3-h food intake in CTR rats. Conversely, icv administration of the orexigenic compound did not modify (vs vehicle-injected values) the amount of food eaten, in 3 h, by MSG-damaged rats.

Discussion

Our results strongly support that hypothalamic actions of orexin A on food intake and HPA axis function operate by independent hypothalamic mechanisms. Although acute icv orexin A–stimulated food intake seems to be dependent on full hypothalamic NPY-ergic activity, its effect on HPA axis function develops even without full ARC nucleus function.

Our data indicate that hypothalamic prepro-orexin, the single protein precursor of orexin A and B, mRNA expression remained normal in adult female rats neonatally treated

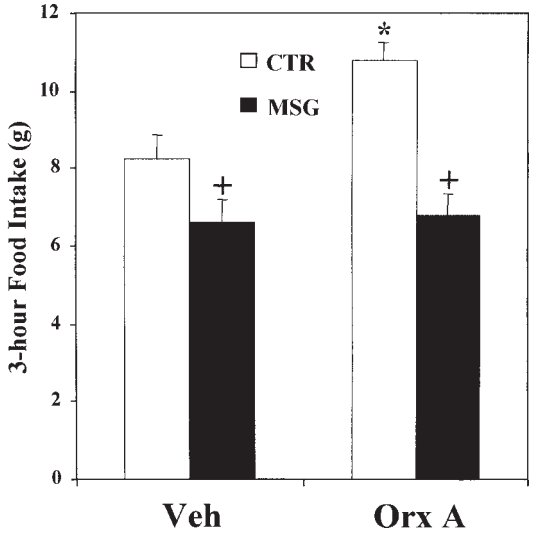

Fig. 5. Food intake, during 3 h, after icv administration of 2 µL of vehicle alone (Veh) or containing 1 µg of orexin A (Orx A) to CTR and MSG rats. Experimentation was performed at 09:00 hours on d 21 of the food-restriction protocol. After rats were bled at time zero, they have had free access to food for 3 h. Values are the mean ± SEM, n = 6–8 rats per group. $^{*}p < 0.05$ vs values from Veh-injected rats of the same group. $^{+}p < 0.05$ vs CTR values.

with MSG. Orexin A, a 33-amino-acid residue peptide, is mainly synthesized in the posteriolateral hypothalamus and perifornical area *(4)* and has, among other hypocretins, the greatest potency at orexin 1 and 2 receptors (OX1R and OX2R, respectively) *(4,21,22)*. The terminals of orexin A neurons project to, among others, both the PVN *(23–25)*, a pivotal structure controlling HPA axis function, and the ARC *(26)*, the main structure regulating food intake. Interestingly, OX1R and OX2R are expressed in the PVN *(27)* and the ARC *(7)*. These latter observations support that orexin A hypothalamic mechanisms of action could be developed by impacting on both PVN and ARC nuclei neurons, thereafter, afferent signals from these structures are responsible for controlling energy balance *(1)*.

Our data contribute evidence regarding the effect of orexin A on the control of HPA axis activity. They could indicate that, even after a significant reduction in hypothalamic NPY-ergic activity, orexin A is capable of developing full HPA axis response. In our experimental design, we used a food-restriction paradigm *(28)* that, like prolonged fasting *(29)*, could help to activate both the hypothalamic orexin A–ergic system and its postsynaptic structures in a model characterized by a lack of intact hypothalamic NPY-ergic but normal prepro-orexin activities. This protocol, supported by a strong hypothetical background, could be beneficial for revealing potential postsynaptic hypothalamic effects of orexin A action *(29)*. Another advantage of our food-restriction protocol is the consequence of reversing hyperleptinemia in MSG rats, because newly established normal leptin levels could impact *(30)* the hypothalamic orexin A system *(5)*, thus balancing both orexin A neuronal expression and post-synaptic effects. Some reports provide supporting evidence that hypothalamic orexin A effect on food intake involves both leptin-sensitive and leptin-insensitive pathways *(31)*. In parallel, and as observed after withdrawal of food *(29)*, different hypothalamic target sites for orexin A could now be sensitized as a consequence of long-term food restriction *(32)*, thus allowing the full operation of exogenously (icv) administered orexin A. In these circumstances, we now report that, in MSG-damaged female rat, orexin A did develop a HPA axis response without any change in food intake. Because it has been suggested that in rats the hypothalamic NPY-ergic may play a key role in modulating orexin A–induced food intake *(33,34)*, we now provide evidence for sustaining that the integrity of NPY neuronal activity is essential for orexin A stimulation of food intake.

Because MSG rats eat less compared to controls, we cannot discard that in this model food intake could be controlled, directly or indirectly, by other hypothalamic systems. However, the hypothalamic lesion induced by neonatal MSG treatment has been claimed to include also the dysfunction of other orexigenic signals. In fact, the ARC hypothalamic cocaine- and amphetamine-regulated transcript *(35)* and agouti-related protein *(36)* systems have been reported to be significantly reduced in MSG-lesioned rodents. However, as we found in the present study, hypothalamic prepro-orexin mRNA expression remains normal in MSG-damaged rats. Thus, in this model, endogenous orexin A could be capable of modulating food intake by acting on different hypothalamic orexin A–receptive sites involved in the control of energy homeostasis *(37)*.

Regarding HPA axis function in MSG-lesioned rats, there are several reports indicating that the HPA axis of these animals respond in vivo to several stressors *(17,38)* including, as our results demonstrate, to orexin A icv administered. Our data further indicate that the orexin A stimulatory effect on HPA axis function could be developed even in the absence of full hypothalamic NPY-ergic activity; thus supporting an ARC-independent mechanism of orexin A action on HPA axis. The international literature provides little evidence on the involvement of NPY fibers in stimulating PVN CRH output *(39)*; only one published study gives a clue for suspecting an NPY-mediated effect of orexin A on enhancement of HPA axis activity *(11)*. This discrepancy could be explained, at least in part, by the possibility that the NPY-antagonist *(11)*, used for those in vitro experiments with hypothalamic explants, could be altering some other, nonspecific, hypothalamic mechanism(s), e.g., the CRH-ergic activity itself. Furthermore, the in vivo study reported by Samson et al. *(12)* determined that activation of HPA axis function took place after icv administration of orexin A, and that, through a very specific in vitro paradigm, orexin A directly depolarizes magno- and parvo-cellular PVN neurons. These data agree with our observations that orexin A effect on HPA axis function seems to take place on the

PVN. However, though not a specific aim of the present study, orexin A was found to modulate body energy not only through hypothalamic effects. Orexin A is recognized able to directly impact both corticotroph *(40)* and adrenocortical *(41)* cells, thus contributing with its hypothalamic effect to enhance circulating glucocorticoid levels, although whether this additional mechanism could result of physiological relevance remains unclear *(42)*.

Finally, and regarding HPA axis function in MSG-damaged rats, the exacerbated HPA axis response developed by MSG rats, regardless of being hyper- or eu-leptinemic, to orexin A icv treatment agree with published data from our group and several others. In fact, it is recognized that hyperleptinemic MSG animals develop enhanced hypothalamic vasopressin and CRH and pituitary ACTH *(43)* secretions. Also, they are characterized by enhanced corticoadrenal function *(15,44–46)* and a loss of the circadian pattern of glucocorticoid secretion *(45)*, abnormalities that seem to be associated with both a reduced glucocorticoid metabolic clearance rate in MSG rats *(45,47)* and a resistance to the inhibitory tone of circulating leptin on the adrenal gland *(15)*. In the present and a previous study from our laboratory *(15)*, we found no changes in basal circulating levels of ACTH in hyperleptinemic MSG rats. Whether the time elapsed with lower leptin circulating levels in food-restricted MSG rats could be able to decrease leptin inhibition of hypothalamic CRH activity *(48)*, thus allowing hypersecretion of CRH (as seen in isolated hypothalamic explants, see ref. *43*) to orexin A stimulus, and thus inducing in turn corticotroph hyperresponse, remains to be determined.

In summary, our study strongly suggests that hypothalamic effects of orexin A on food intake and HPA axis function take place by independent mechanisms of action. While orexin A–induced food intake needs a full hypothalamic NPY-ergic activity, conversely, this neuropeptide is able to trigger HPA axis response in a full NPY-ergic–independent manner. Moreover, from a physiological point of view, the fact that orexin A independently stimulates food intake and CRH could indicate that, at least partially, enhanced CRH *(1)* activity could be then protecting the organism from excess food intake, thus maintaining homeostasis.

Materials and Methods

Animals and Treatment

Adult male (300–330 g BW) and female (240–280 g BW) Sprague–Dawley rats were allowed to mate in colony cages in a light (lights on from 07:00 to 19:00 h)- and temperature (22°C)-controlled room. Rat chow and water were available *ad libitum*. Pregnant rats were transferred to individual cages. Beginning on d 2 after parturition, newborns were injected ip with either 4 mg/g BW monosodium L-glutamate (MSG; Sigma Chemical Co., St. Louis, MO) dissolved in sterile 0.9% NaCl or 10% NaCl (littermate controls; CTR) once every 2 d up to d 10 of age *(43)*. Rats were weaned and sexed at 21 d of age; daily body weight and food intake of female rats were recorded up to the experimental day (120 d of age). Although our CTR and MSG animals, eating *ad libitum*, have already been characterized by a reduced hypothalamic NPY mRNA expression *(15)*, in the present experiments MSG-injected animals were screened for effectiveness of treatment by macroscopic observation of degeneration of the optic nerves and the reduced hypothalamic NPY mRNA expression (see Results) at the time of sacrifice. Additionally, hypothalamic prepro-orexin mRNA expression was semiquantified (see below). In each experiment, CTR and MSG female rats were members of the same litters; however, when accumulating experiments, each different experiment was performed with (CTR and MSG) animals from different litters. Because MSG rats had an abnormal estrous cycle, the microscopic observations of their daily vaginal smears demonstrating a constant diestrous stage, we have used CTR litters for experimentation when screening showed that they were at the diestrous stage of their estrous cycle. Our Animal Care Committee approved all experiments. Animals were killed by decapitation, according to protocols for animal use, in agreement with NIH Guidelines for care and use of experimental animals.

Experimental Designs

One hundred and twenty day old, CTR and MSG, female animals (10–12 rats per group) were weighed, caged individually, and subjected or not to a food-restriction protocol *(28)*. Animals submitted to food restriction were weighed (08:30 hours) and allowed to eat *ad libitum* (by adding 30 g of Purina chow diet per cage) every day between 09:00 and 12:00 hours; after this hour, the remaining food was withdrawn from the cage and weighed. The amount of food eaten in a 3-h period was calculated by subtracting grams of remaining food from 30 g. BWs and 3-h food intake were recorded daily from d 1 up to the experimental day (day 21 after the beginning of this food restriction protocol). On d 14 of the food-restriction protocol, rats from both groups were implanted, under light ketamine anesthesia, with both icv (coordinates from the point of bregma, in mm, were 1.3 posterior, 0.4 lateral, and 3.8 deep) and iv catheters. On d 21, rats were bled (300 μL) before and several times (15–180 min) after icv injection of 2 μL of vehicle alone (sterile saline solution) or containing 1 μg of orexin A (Sigma); immediately after the icv treatment, 30 g of food was provided to each rat. Blood volume taken at each bleeding time was replaced by blood cells resuspended in sterile saline solution; however, the same volume of sterile saline solution replaced the time-zero sample only. At the end of experimentation, the remaining food in the cage was recorded for each animal; thereafter, rats were sacrificed for microscopic observation of optic nerves and determination of hypo-

thalamic NPY mRNA expression. In each experiment, with animals eating *ad libitum* or food-restricted, 6–8 rats per group (CTR and MSG), condition (vehicle and orexin A) were used. Blood samples were immediately centrifuged and plasma samples kept frozen (–80°C) until further determination of plasma concentrations of ACTH, corticosterone (B), and leptin.

Hypothalamic RNA Extraction and RT-PCR Analysis

Total RNA was isolated from hypothalami for semiquantification of NPY and prepro-orexin mRNAs expression. Hypothalamic tissues were dissected as previously reported *(49)*; limits: posterior border of the optic chiasm, anterior border of the mamillary bodies, and lateral hypothalamic border, 3 mm deep (approximately). Tissue RNA extraction was done by using a modification of the single-step, acid guanidinium isothiocyanate–phenol–chloroform extraction method described by Chomzynski et al. *(50)* (Trizol; Invitrogen, Life Tech., USA; cat. no. 15596-026). The yield and quality of extracted RNA were assessed by 260/280 nm optical density ratio and electrophoresis, under denaturating conditions, on 2% agarose gel. One microgram of total RNA was incubated with 0.2 m*M* dNTPs, 1 m*M* $MgSO_4$, 1 μ*M* of specific primers (see below), 1 μ*M* β-actin primers, 0.1 U/μL AMV reverse transcriptase (5 U/μL), 0.1 U/μL Tfl DNA polymerase (5 U/μL); final volume of 25 μL. Amplifications were done in a thermal cycler (Perkin-Elmer) under the following conditions: 48°C–45 min for reverse transcription step (1 cycle); 94°C–2 min for AMV reverse transcriptase inactivation and RNA/cDNA/primers denaturation (1 cycle); 94°C–30 s for denaturation; 54°C–1 min and 60°C–1 min, NPY and prepro-orexin respectively, for annealing; 68°C–2 min for extension (40 cycles); 68°C–7 min for final extension (1 cycle); and 4°C for soak (Promega Access RT-PCR System No. A1250). NPY primers *(15)* were designed for a high homology region of the NPY gene: (F) 5'- CCC GCC ATG ATG CTA GGT AAC -3' and (R) 5'-ACA AGG GAA ATG GGT CGG AAT -3' (430 bp) (GenBank accession number: NM012614). Prepro-orexin primers *(51)* were designed for a high homology region of the PPOrx gene: (F) 5'- AGA CTC CTT GGG TAT TTG GAC -3' and (R) 5'-TAA AGC GGT GGC GGT TGC AGT -3' (400 bp) (GenBank accession number: NM013179). In this semiquantitative technique, the third set of primers was specific for the β-actin gene, with the following sequences: (F) 5'-TTG TCA CCA ACT GGG ACG ATA TGG-3' and (R) 5'-GAT CTT GAT CTT CAT GGT GCT AGG-3' (764 bp) (GenBank accession number: NM031144). Controls without reverse transcriptase were systematically performed to detect cDNA contamination. Amplified products were analyzed on 2% agarose gel and visualized by ethidium bromide UV transillumination in a Digital Imaging System (Kodak Digital Science, Electrophoresis Documentation and Analysis 120 System).

Hormones Determinations

Circulating ACTH concentrations were measured by a previously described immunoradiometric assay *(49)*, the standard curve ranged between 15 and 3,000 pg/mL and intra- and interassay coefficients of variation (CVs) were 2–3 and 6–8%, respectively. Plasma B concentrations were evaluated by a specific radioimmunoassay (RIA) reported earlier *(49)*, the standard curve ranged between 1 and 250 μg/dL, and intra- and interassay CVs of were 4–6 and 8–10%, respectively. Leptin circulating levels were determined by a specific RIA from our laboratory (previously validated for rat leptin, see ref. *52*); the standard curve ranged between 0.2 and 25 ng/mL, CVs intra- and interassay were 5–8 and 10–12%, respectively.

Analysis of Data

Data (mean ± SEM) for circulating hormone concentrations were analyzed by ANOVA with repeated measurement, followed by Student–Newman–Keul's test for comparison of different mean values. The AUCs for circulating hormones were obtained using the trapezoidal rule. Finally, the nonparametric Mann–Whitney test was used for analysis of data from hypothalamic neuropeptide mRNAs expressions *(53)*.

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